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Sequences encoding a kin17 protein and uses thereof

The present invention relates to the cDNA sequence encoding the human kin17 protein, to the cDNA sequences encoding a truncated kin17 protein, termed kin17 $\Delta$ HR, as well as to the use of said nucleic acid sequences and said proteins in the regulation of cell proliferation.

The present invention also relates to a method for detecting the human *Kin17* gene and the mRNA of the human *Kin17* gene by *in situ* hybridization with the aid of oligonucleotides and/or by polymerase chain amplification (PCR).

The present invention also relates to expression vectors or plasmids which express the abovementioned proteins and to the bacteria containing said vectors or plasmids.

The present invention also relates to the use of said vectors for producing and purifying the kin17 protein and truncated or modified forms thereof, and as a medicinal product (gene therapy).

A protein, termed kin17, has been demonstrated in mice by J.F. Angulo et al. (*Mutation Res.*, 1989, 123-134), and is immunologically related to the recA protein of *E. coli*; its identification has been possible, with the aid of anti-*E. coli* recA antibodies, in FR 3T3 rat cells. The production of this kin17 protein is induced by genotoxic agents, such as ultraviolet radiation. It is preferentially located in the nucleus. In addition, it is a minority cellular protein, and is very sensitive to enzymatic proteolysis.

It is probably involved in DNA metabolism, since it increases during certain cellular phases of DNA synthesis, and in repair, since it accumulates in the nucleus after DNA modification.

It has been shown (D.S.F. Biard et al., *Radiation Research*, 1997, 147, 442-450) that the

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expression of this kin17 protein in rats is increased in the presence of ionizing radiations, and that it intervenes in DNA repair (A. Tissier et al., *Genomics*, 1996, 38, 238-242).

Continuing with their work, J.F. Angulo et al. (*Biochimie*, 1991, 73, 251-256) characterized monospecific anti-recA antibodies, which can be used for detecting the expression of said kin17 protein by expression vectors. These authors have thus highlighted a cDNA fragment, termed *Kin17*<sub>601</sub>, which is derived from mouse embryonic RNA and which expresses a polypeptide (*kin17*<sub>200</sub>) which cross-reacts with the anti-recA antibodies.

The comparison of the sequence of the polypeptide (*kin*<sub>200</sub>) encoded by this cDNA with the sequence of the recA protein has led to the demonstration of common sequences which correspond to sequences located between amino acids 309 to 347 of recA.

In *Nucleic Acids Research* (1991, 19, 5117-5123), J.F. Angulo et al. identified and expressed the *Kin17* cDNA (1414 bp) in mice, using the abovementioned *Kin17*<sub>601</sub> fragment as probe.

The mouse *Kin17* cDNA presents a single open reading frame, between positions 25 and 1198, which encodes a 391-amino acid protein (protein *mkin17*) which presents a zinc-binding domain ("zinc finger" motif) between residues 28 and 50 and an antigenic determinant of the same type as that of the recA protein between positions 162 and 201.

This protein presents a nuclear localization signal, located between positions 240 and 256, which appears to be similar to those identified in some nuclear proteins, and which is functional.

The chromosomal localization of the gene encoding the murine kin17 protein has been performed by *in situ* hybridization, and found to be on chromosome 2 in mice. The human *Kin17* gene is located on chromosome 10p15-p14.

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The "zinc finger" motif is involved in the binding of kin17 protein to double-stranded DNA.

kin17 protein binds preferentially to curved DNA; its binding efficiency is correlated with the extent of curving of the DNA.

Two proteins having various modifications (deletions), either in the "zinc finger" motif (kin17  $\Delta$ 1) or at the C-terminal end of the protein (kin17  $\Delta$ 2), conserve the property of preferential binding to curved DNA; these properties show that the "zinc finger" motif is not essential for preferential binding to this curved DNA, and that another domain which recognizes curved DNA is involved (Mazin et al., N.A.R., 1994, 22, 20, 4335-4341) and is located between amino acids 71 and 281.

Continuing with their work, J.F. Angulo et al., have also shown the presence of kin17 protein in mammals other than mice, and in particular in humans. For example, D.S.F. Biard et al. (*Arch. Dermatol. Res.*, 1997, 289, 448-456), using anti-mouse kin17 antibodies, have detected the human kin17 protein ( $_{HS}$ kin17) in skin cells and have shown that the levels of  $_{HS}$ kin17 protein increase in epithelial keratinocytes in the proliferation phase (after 7 days of culture), whereas these levels fall in the differentiation phase.

However, despite the detection of the  $_{HS}$ kin17 protein, it has not been possible, hereto, to effectively isolate the coding sequence of this human protein, not even using probes of murine origin.

For example, the nucleic acid fragment, of human origin, described in French patent No. 2,706,487 does not make it possible to express the human kin17 protein, or to isolate the complete nucleic acid sequence which is capable of an effective expression of this  $_{HS}$ kin17 protein.

The inventors have now found, surprisingly, that the expression of the mammalian (in particular mouse and human) kin17 protein is in general correlated with cell proliferation; they have in particular found

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that the kin17 protein inhibits cell proliferation; in addition, an overexpression of kin17 protein or of a C-terminal fragment of said kin17 protein drastically inhibits cell proliferation.

They have also found that a truncated protein (deletion of a fragment comprising the region which is homologous (HR) between the kin17 protein and the recA protein) was even more active in inhibiting cell proliferation.

Consequently, the inventors set themselves the aim of providing medicinal products which are capable of regulating cell proliferation, based on these sequences.

A subject of the present invention is a nucleic acid sequence, characterized in that it presents the sequence SEQ ID NO. 1 and in that it is capable of expressing a functional human kin17 protein.

A subject of the present invention is also a nucleic acid sequence, characterized in that it encodes a kin17 protein which is truncated at the region which is homologous to the recA protein.

According to an advantageous embodiment of said nucleic acid sequence, it encodes a truncated kin17 protein which corresponds to a kin17 protein in which at least the fragment between amino acids 162 and 201, and at most the fragment between amino acids 55 to 235, is deleted.

According to an advantageous arrangement of this embodiment, said nucleic acid sequence encodes a truncated kin17 protein which corresponds to the mouse kin17 protein in which the fragment between amino acids 129 to 228 is deleted, and which presents the sequence SEQ ID NO. 2.

According to another advantageous arrangement of this embodiment, said nucleic acid sequence encodes a truncated kin17 protein which corresponds to the human kin17 protein in which the fragment 129 to 228 is deleted, and which presents the sequence SEQ ID NO. 3.

A subject of the present invention is also

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fragments, of 20 to 40 nucleotides of the sequence SEQ ID NO. 1, for detecting the gene encoding the human kin17 protein, and/or the RNA of the *Kin17* gene, in a biological sample.

Such fragments are used in particular as primers for PCR, RT-PCR or *in situ* hybridization. Depending on the case, they can advantageously be labelled with the aid of a suitable nonradioactive label (fluorescent substances, ligands such as biotin or a hapten).

Among said fragments, mention may be made of in particular the sequences SEQ ID NO. 5 to 21, listed in the table below, as well as the sequences SEQ ID NO. 33 and 34.

Hu89D (SEQ ID NO. 5)	CTCAGGAGACGCTTTGGCACTA
Hu857R (SEQ ID NO. 6)	CCTGGTGCTGGAATTACTGTCT
Hu3r (SEQ ID NO. 7)	TCTTTTCGTTTCACTGATGCT
Hu4d (SEQ ID NO. 8)	GGGAGAGAAATATCATAAGAAAA
Hu5d (SEQ ID NO. 9)	TCCCTCTGTAGCCCTCCCATT
Hu6d (SEQ ID NO. 10)	TTTTTCAGCTACTATCGTTCATT
Hu8d (SEQ ID NO. 11)	CGAGTGCCTGAAGACGATAGG
Hu9r (SEQ ID NO. 12)	ATTCTTTTCGTTTCACTGAT
Hu10r (SEQ ID NO. 13)	GGCAATACCAGCGTAGCTTCTGCAGC
Hu11r (SEQ ID NO. 14)	CTCTGATGAGATTCCGGACATACAAT
Hu12r (SEQ ID NO. 15)	TCTCCTGAGAAGTTCTAGAAA
Hu-KPNd (SEQ ID NO. 16)	ACTGCCAAATTTATTGAAGAGCAAGTGAGAAG AGGCCTGG
Hu-KPNr (SEQ ID NO. 17)	CCAGGCCTCTTCTCACTTGCTCTTCAATAAAT TTGGCAGT
HsKin10d (SEQ ID NO. 18)	AGAAAGTGATCGCTGCCGTGGT
HsKin1251r (SEQ ID NO. 19)	GCGAACACCAATTTGATGCTTTAAGA
Hu174D (SEQ ID NO. 20)	TCAGAGACAACCTATTGCTGGC
Hu1170R (SEQ ID NO. 21)	ATTCTTCAACTCTGCGTCCTT

A subject of the present invention is also fragments of the sequence SEQ ID NO. 1 which can be used as probes for DNA-DNA or DNA-RNA hybridization.

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Among such fragments, mention may be made of the sequence SEQ ID NO. 4, which corresponds to positions 207 to 1208 of the sequence SEQ ID NO. 1 (probe 1000), and the sequences SEQ ID NO. 1 and 5 to 21.

A subject of the present invention is also fragments of a nucleic acid sequence encoding a segment of a mammalian kin17 protein (sequences SEQ ID NO. 1 and SEQ ID NO. 24), these fragments comprising between 300 and 360 nucleotides encoding the C-terminal portion of said kin17 protein, and being capable of controlling cell proliferation.

According to an advantageous embodiment of said fragments, they are selected from the group consisting of SEQ ID NO. 33 and SEQ ID NO. 34.

A subject of the present invention is also a method for detecting genomic DNA or a transcription product of the human *Kin17* gene, by gene amplification and/or hybridization, which is carried out starting from a biological sample, this method being characterized in that it comprises:

(1) a step in which a biological sample to be analysed is brought into contact with at least one probe selected from the group consisting of the sequences SEQ ID NO. 1 to 21 and

(2) a step in which the resulting product(s) of the nucleotide sequence-probe interaction is (are) detected by any suitable means.

In accordance with said method, it can comprise, prior to step (1):

. a step for extracting the nucleic acid to be detected, and

. at least one gene amplification cycle carried out with the aid of a pair of primers selected from the sequences SEQ ID NO. 5 to 21.

According to an advantageous embodiment of said method, the probe in step (1) is optionally labelled with the aid of a label such as a radioactive isotope, an appropriate enzyme or a fluorochrome.

According to an advantageous arrangement of

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this embodiment, said probe consists of the sequence SEQ ID NO. 4.

According to another advantageous embodiment of said method, said pair of primers consists of a sequence SEQ ID NO. 16 paired with a sequence SEQ ID NO. 17.

A subject of the present invention is also a method for detecting a transcription product of the human *Kin17* gene, characterized in that it comprises:

- a step for extracting the RNA to be detected,
- a step for synthesizing the cDNA corresponding to said RNA by reverse transcription in the presence of random primers,
- at least one gene amplification cycle carried out with the aid of a pair of primers selected from the sequences SEQ ID NO. 5 to 21, and
- the detection of the amplified product.

According to an advantageous embodiment of said method, said pair of primers is selected from the group consisting of the following pairs: sequences SEQ ID NO. 5 and SEQ ID NO. 12, for amplifying a 453-bp fragment (fragment A); sequences SEQ ID NO. 18 and SEQ ID NO. 19, for amplifying a 1265-bp fragment (fragment B) and sequences SEQ ID NO. 16 and SEQ ID NO. 7, for amplifying a 224-bp fragment (fragment C).

Advantageously, the amplified cDNA fragments are separated by electrophoresis, preferably on agarose gel, visualized in the presence of ethidium bromide and quantified with the aid of the NIH image program (National Institute of Health, USA).

In accordance with the invention, it also encompasses the reagents for detecting a nucleic acid sequence encoding a mammalian *kin17* protein or a modified fragment of these sequences, characterized in that they include the sequences SEQ ID NO. 4 to 21, 33 and 34, as well as the fragments A of 453-bp, B of 1265-bp and C of 224-bp, optionally labelled.

A subject of the present invention is also a *kin17AHR* protein, characterized in that it corresponds

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to a kin17 protein which is truncated at a region which is a homologue of the recA protein.

According to an advantageous embodiment of said truncated kin17 $\Delta$ HR protein, it corresponds to a kin17 protein in which at least the fragment between amino acids 162 and 201, and at most the fragment between amino acids 55 to 235, is deleted.

According to an advantageous arrangement of this embodiment, said truncated kin17 $\Delta$ HR protein corresponds to the mouse kin17 protein in which the fragment between amino acids 129 to 228 is deleted, and presents the sequence SEQ ID NO. 22 (sequence termed  $m_m$ kin17 $\Delta$ HR).

According to another advantageous arrangement of this embodiment, said truncated kin17 $\Delta$ HR protein corresponds to a human kin17 protein in which the fragment 129 to 228 is deleted, and presents the sequence SEQ ID NO. 23 (sequence termed  $h_s$ kin17 $\Delta$ HR).

A subject of the present invention is also fragments of kin17 protein, characterized in that they comprise between 100 and 120 amino acids, and are located in the C-terminal position; they are preferably selected from the group consisting of SEQ ID NO. 35 and SEQ ID NO. 36.

Unexpectedly, such fragments inhibit cell proliferation; the sequence SEQ ID NO. 35 corresponds to residues 283 to 393 of the human sequence of kin17 (SEQ ID NO. 26); the sequence SEQ ID NO. 36 corresponds to residues 281 to 391 of the mouse sequence of kin17 (SEQ ID NO. 25).

A subject of the present invention is also the use of the fragment between amino acids 55 to 235, optionally mutated, preferably the fragment between amino acids 129 and 228, of a mammalian kin17 protein, for regulating the protein-curved DNA interaction.

In fact, the production of mutants in this protein-curved DNA interaction domain constitutes a tool of choice for blocking certain biological processes, such as proliferation, translation or the



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integration of the AIDS virus into the human genome, or for transporting effector proteins by constructing curved DNA binding domain-repair enzyme protein fusions, at sites where the DNA is curved.

A subject of the present invention is also the use of a mammalian kin17 protein or of a 100- to 120-amino acid C-terminal fragment of said kin17 protein for preparing a medicinal product which regulates cell proliferation or fertility.

In accordance with the invention, said mammalian kin17 protein or said 100- to 120-amino acid C-terminal fragment is used for preparing a medicinal product which inhibits cell proliferation, and which is in particular intended for treating diseases in which a cellular hyperproliferation is observed.

According to an advantageous embodiment of said use, said sequence is selected from the group consisting of the sequences SEQ ID NO. 22, 23, 25, 26, 35 and 36.

A subject of the present invention is also an expression vector, characterized in that it includes a sequence encoding a mammalian kin17 protein or a fragment of it selected from the group consisting of the sequences SEQ ID NO. 1, 2, 3, 33 and 34.

According to an advantageous embodiment of said vector, said sequence encoding said kin17 protein or said fragment of it is fused with a gene which encodes a fluorescent protein.

Such vectors are in particular useful:

- for preparing a medicinal product which controls cell proliferation,
- as a detection tool, in particular for visualizing the sites and the progression of DNA repair, and the intranuclear centres of biosynthesis.

Advantageously, said vector is combined with appropriate regulatory sequences.

A subject of the present invention is also the use of an expression vector which includes a sequence selected from the group consisting of the sequences SEQ

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ID NO. 1, 2, 3, 24, 33 and 34, for preparing a medicinal product which controls cell proliferation.

Preferably, said vector is a plasmid; it can be maintained in bacteria such as *E. coli*; by way of example, mention may be made of the bacterium MOSBlue (Amersham, France), which possesses the following genotype:

end AI hsdR17 (<sup>x</sup><sub>k12</sub>-<sup>m</sup><sub>k12</sub>+) sup E44 thi-1 rec AI gyr A96 rel AI lac [F' pro A<sup>+</sup>B<sup>+</sup> lac<sub>q</sub>I<sup>q</sup>2ΔM15::Tn10(Tc<sup>R</sup>)].

Transformed CMOS bacteria are thus obtained:

- PK1 bacterium: CMOS bacterium transformed with the plasmid pMOSBlue (Amersham, France) into which has been introduced the cDNA *<sub>HS</sub>Kin17* (defined by SEQ ID NO. 1);

- PK2 bacterium: CMOS bacterium transformed with the plasmid pCMVDT21 (Bourdon et al., 1997, Oncogene) into which has been introduced the cDNA *<sub>HS</sub>Kin17* (defined by SEQ ID NO. 1).

Besides the above arrangements, the invention comprises other arrangements, which will emerge from the description which will follow, and which refer to examples of use of the method which is the subject of the present invention, as well as to the attached drawings, in which:

- Figure 1 illustrates the detection of the PCR products by gel electrophoresis. The DNA is revealed by ethidium bromide staining. DNA fragments of known size are present in column A, as molecular weight markers.

Q1 - Figures 2A and 2B illustrate the comparison of the nucleic acid (Figure 2A) and protein (Figure 2B) sequences *<sub>HS</sub>Kin17* and *<sub>Mm</sub>Kin17*. Nucleotides = 86% identity, amino acids = 92.4% identity.

- Figure 3 represents an autoradiograph of the hybridization analysis of the total RNAs extracted from various human tissues (panel A) or from various human tumour cells (panel B), using the probe-1000 (SEQ ID NO. 4).

- Figure 4 illustrates the detection of the messenger RNA of the *Kin17* gene in mouse testicle, by

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*in situ* hybridization.

- Figure 5 is a schematic representation of the proteins produced by transient transfection. The amino acid sequence is represented linearly. The name of the proteins is indicated to the left of each protein, and the size is mentioned to the right.

- Figure 6 illustrates the detection of the HeLa cells transfected with the vector pCMV<sub>Mm</sub>Kin17. The nuclei are stained in blue using DAPI. The green intranuclear coloration corresponds to the location of the <sub>Mm</sub>kin17 protein; Figure 6A illustrates the detection of <sub>Mm</sub>kin17 in cells which express a low level of <sub>Mm</sub>kin17 protein and Figure 6B illustrates the detection of <sub>Mm</sub>kin17 in cells which express a high level of kin17 protein; magnification: 1000.

- Figure 7 represents the HeLa cells which express a low level of <sub>Mm</sub>kin17 protein (with intranuclear foci having a diameter of approximately 0.5  $\mu$ m; panel A) or cells which express a high level of kin17 protein (detection of nuclear morphology deformations NMD; panel B).

- Figure 8 represents the immunodetection of the <sub>Mm</sub>kin17 $\Delta$ HR protein in the HeLa cells transfected with the plasmid pCMVKin17 $\Delta$ HR. Use of pAbanti-recA as first antibody (panel A). Detection with the antibody pAb2064 (panel B).

- Figure 9 corresponds to the analysis by immunocytochemistry and by phase contrast microscopy of the HeLa cells which overproduce the <sub>Mm</sub>kin17 $\Delta$ CT protein.

- Figure 10 shows that the nuclear morphology deformation is correlated with an inhibition of the DNA replication. Figure 10A represents the immunodetection of the <sub>Mm</sub>kin17 $\Delta$ HR protein (in red) and the incorporation of BrdU (in green) in the HeLa cells expressing the <sub>Mm</sub>kin17 $\Delta$ HR protein. BrdU (bromodeoxyuridine) is a nucleotide (thymidine) analogue which incorporates into DNA during replication. Figure 10B is a summary table which shows the inhibition of DNA replication after formation of nuclear morphology deformations (NMD), due

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to the expression of the  $Mm$ kin17 $\Delta$ HR or  $Mm$ kin17 proteins.

- Figure 11 illustrates the genetic map of the plasmids pEBVMTA (pB220) and pEBVMT $Mm$ kin17 (pB223).

- Figure 12 illustrates the laser scanning cytometry analysis of the  $Mm$ kin17 protein in the cells of the clone B223.1. 9 months after the transfection, both the B223.1 cells and the B220 cells are seeded in a proportion of  $3 \times 10^4$  cells/cm<sup>2</sup>, three days before beginning the treatment with heavy metals (100  $\mu$ M ZnCl<sub>2</sub> and 1  $\mu$ M CdSO<sub>4</sub>). 24 hours later, the cells are fixed and stained with an anti-recA antibody specific for the  $Mm$ kin17 protein. The immunocytochemical detection is carried out using the ACAS 570 cytometer (Meridian Inc.). Panels A and B correspond to the B223.1 cells, with or without heavy metals; panels C and D correspond to the B220 cells, with or without heavy metals. Each section is analysed for the  $Mm$ kin17 protein (left-hand panel) and propidium iodide (PI) (right-hand panel) at the same time. The intensities of fluorescence specific for the  $Mm$ kin17 protein and for PI are represented using arbitrary scales of fluorescence.

- Figure 13 illustrates the immunocytochemical detection of the  $Mm$ kin17 protein in the cells of the clone B223.1: 6 months after the transfection, the B223.1 and B220 cells are seeded in a proportion of  $6 \times 10^4$  cells or  $10^4$  cells/cm<sup>2</sup> respectively, and treated under the same conditions as those set out for Figure 12. The staining is carried out with the anti-recA antibody. Panels A and B correspond to the B223.1 cells, with or without heavy metals; panels C and D correspond to the B220 cells, with or without heavy metals. An anti-recA antibody and a secondary antibody conjugated to the fluorochrome Cy2<sup>TM</sup> are used to detect the  $Mm$ kin17 protein. The fluorescence is analysed using a Visiolab 1000 program (Biocom) coupled to an Axiphot 2 microscope (Zeiss) and a cooled camera, as specified above (magnification of each panel: 100).

- Figure 14 shows polynucleated B223.1 cells overexpressing the  $Mm$ kin17 protein: 8 months after the

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transfection, B223.1 cells are seeded as specified above (see Figure 12). 24 hours after the treatment with heavy metals ( $100 \mu\text{M ZnCl}_2$  and  $1 \mu\text{M CdSO}_4$ ), these cells are fixed and stained with an anti-recA antibody. Magnification: (A) 504; (B and C) 1000; (D and F) 1260; (E) 2000. The fluorescence is analysed using a Visiolab 1000 program.

- Figure 15 illustrates the cell cycle analysis in HEK293 cells overexpressing the  $\text{mmkin17}$  protein: 6 months after the transfection, the cells are seeded at the same dilutions and, three days later, a treatment with heavy metals for 24 hours. The cells are recovered and analysed. The arrows indicate the polynucleated cells.

- Figure 16 illustrates the influence of the overproduction of the  $\text{mmkin17}$  protein on the efficiency of the B223.1 cells in forming clones: 8 months after the transfection, the B220, B223.1 and B223.2 cells are seeded at  $10^3$ ,  $10^2$  and  $10^1$  cells/cm<sup>2</sup> in the presence of hygromycin B ( $125 \mu\text{g/ml}$ ). After 10, 12 and 18 days in culture respectively, the cells are fixed and stained.

- Figure 17 illustrates the proliferation rate of the B223.1 cells: at various moments after the transfection, cell growth is evaluated. (A): cells are seeded at the same dilution ( $10^3$  cells/cm<sup>2</sup>) in the presence of hygromycin B ( $125 \mu\text{g/ml}$ ). At various moments after the seeding, the cells are trypsinized and counted. (B): to evaluate the efficiency in forming clones, the three cell lines are evaluated at various densities:  $10^3/\text{cm}^2$  for the B220 cells,  $2 \times 10^3/\text{cm}^2$  for the B223.2 cells and  $10^4/\text{cm}^2$  for the B223.1 cells. The experiments are carried out in the presence or absence of hygromycin B ( $125 \mu\text{g/ml}$ ). For each curve, the mean of three culture dishes is calculated: -O-: B220 cells without hygromycin; -●-: B220 cells with hygromycin; -□-: B223.1 cells without hygromycin; -■-: B223.1 cells with hygromycin; -p-: B223.2 cells without hygromycin; -v-: B223.2 cells with hygromycin.

- Figure 18 illustrates the *in vivo* detection,

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by fluorescence, of the fusion proteins GFP-kin17ACT (to the left) and GFP-kin17NLS-CT (to the right) after transfection of HeLa cells with the plasmids pEGFP-Kin17ACT and pEGFP-Kin17NLS-CT respectively.

In these figures, the various stainings appear in a greyish form.

It should be well understood however that these examples are given only by of illustration of the subject of the invention, of which they in no way constitute a limitation

**EXAMPLE 1: Method for cloning the human *Kin17* gene (*<sub>HS</sub>Kin17*) cDNA.**

To isolate the *<sub>HS</sub>Kin17* gene cDNA, human lymphoblastoid cells, termed Boleth cells, which possess a normal carotype, were used.  $10^7$  cells were treated with a denaturing solution (RNA-B, Bioprobe, France); the proteins were removed after centrifugation at 12,000 rpm for 20 min. at 4°C. The total RNAs are recovered in the aqueous phase and precipitated by addition of one volume of isopropyl alcohol at -20°C and centrifuged. After resuspending in 1 ml of distilled H<sub>2</sub>O, the RNAs are again precipitated at -20°C in one volume of isopropyl alcohol and 0.2 M NaCl. After recovering by centrifugation and rinsing with 70% ethanol, the RNAs are resuspended in water and stored at -80°C. The RNAs thus obtained were subsequently used in the RNA reverse transcription reaction (RT), for synthesizing the corresponding complementary DNAs. The cDNAs obtained are treated by polymerase chain reaction (PCR), in the presence of a heat-stable polymerase, to obtain an *<sub>HS</sub>Kin17* cDNA fragment.

**Production of a 1000-nucleotide fragment of the human cDNA by RT-PCR.**

Oligonucleotides derived from the nucleotide sequence of the mouse *Kin17* cDNA (oligonucleotide pair SEQ ID NO. 27 = TCAAAGACAACTGTTGCTGGC and SEQ ID NO. 28 = ARACCTTCAACTCTGCGTCCTT) were used in the following way: 1 µg of total RNAs was mixed with 5 mM MgCl<sub>2</sub>, 1X PCR buffer, 1 mM dNTPs, 1 U/ml of RNase inhibitor,

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2.5 mM oligo d(T)<sub>16</sub> and 2.5 U/ml of reverse transcriptase. The mixture is incubated for 20 min. at room temperature to allow the oligo d(T)<sub>16</sub> sequence to hybridize with the polyA<sup>+</sup> ends of the RNAs and to initiate the reverse transcriptase. The mixture is then incubated for 20 min. at 42°C, and then for 5 min. at 99°C and for 5 min. at 5°C. These various incubations allow the reverse transcription of the messenger RNAs into complementary DNA. The PCR is carried out using the abovementioned oligonucleotides SEQ ID NO. 27 and 28. After PCR, the amplification products were separated on a 1.5% agarose gel. The presence of DNA is revealed with ethidium bromide (Figure 1). The size markers present in column A of Figure 1 made it possible to determine the size of the amplification products. A 1000-base pair DNA fragment is clearly detectable in the case of an amplification carried out on the cDNA obtained (column C). Conversely, when the enzyme which enables the reverse transcription step is omitted, it is observed that this DNA fragment is absent (column B). This clearly demonstrates the presence of the *hsKin17* messenger RNA in the human cells; the 1000-base pair fragment obtained (SEQ ID NO. 4) is not due to a genomic DNA contamination.

The sequence of the 1000-base pair fragment is determined according to the automatic sequencing technique described in Tissier A. et al., 1996, mentioned above. The sequence SEQ ID NO. 4, which corresponds to an *hsKin17* cDNA fragment, is 86% identical to that of mouse *mmKin17*. The polypeptide encoded is 92.4% identical. It is the isolation of this *hsKin17* cDNA fragment which has effectively made it possible to undertake the cloning of the complete *hsKin17* cDNA (Figure 2: comparison of the sequences of the *hsKin17* cDNA fragments with the mouse *Kin17* cDNA).

**EXAMPLE 2: Screening a human cDNA library: cloning and determination of the nucleotide sequence of the *hsKin17* gene complementary cDNA.**

The radiolabelled 1000-base pair fragment of

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the *HSKin17* gene cDNA (termed probe-1000; SEQ ID NO. 4) was used as probe for screening a human cDNA library obtained from messenger RNAs expressed in testicles and inserted in the vector  $\lambda$ gt11. The DNA of the phages which hybridize with the probe was purified, and the human cDNA was sequenced.

**PROTOCOL:** The library was obtained from polyA<sup>+</sup> RNA purified from human testicles. The complementary DNAs were obtained using a poly d(T) sequence as primer. After reverse transcription and degradation of the RNAs which served as matrix, nucleotide sequences corresponding to the restriction site of the EcoRI digestion enzyme were grafted onto both sides of the cDNA ends. After digestion with the EcoRI enzyme, all the cDNAs are inserted at the EcoRI site of the  $\lambda$ gt11 vector. 250,000 recombinant  $\lambda$ gt11 bacteriophages containing human cDNAs are incubated for 20 min. at 37°C with 0.3 ml of receptor bacteria 6334 (16 h preculture at 37°C), and then mixed with 9 ml of LB agarose medium heated to 48°C. The whole mixture is poured onto Petri dishes of 140 mm in diameter containing LB-agar medium. The dishes are then incubated at 42°C for 5 hours, and then at 37°C for 16 h, until then lysis plaques reach confluence. A dry nitrocellulose filter (Schleicher & Shuell, BA85) is then laid, for 1 min., onto the surface of the dish, which is asymmetrically marked with China ink. In this way, a replica of the lysis plaques is obtained on the filter. The radiolabelled 1000-base pair fragment (probe-1000) (SEQ ID NO. 4) of the *HSKin17* gene cDNA is used for the screening according to the following treatments:

**a) Denaturation and immobilization of the  $\lambda$ gt11 bacteriophage DNA on nitrocellulose filters.**

The filters are placed for 5 min., with the surface which was in contact with the phages facing upwards, onto sheets of Whatmann paper which has been presoaked in denaturing solution (0.5 N NaOH, 1.5 M NaCl), to enable phage lysis and DNA denaturation. The

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filters are then transferred into the neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH = 7.4) for 5 min., then washed for 10 min. in 2X SSC buffer, and then dried at 80°C, under vacuum, for 2 hours. A second series of the filters is brought into contact with the lysis plaques, for 2 min., at the surface of the dish. They will be treated in the same way as the first imprint. The dishes containing the phages are stored at 4°C in the dark. The DNA fixed to the filters is then hybridized with the radiolabelled probe-1000 (Maniatis et al., *Molecular Cloning, a Laboratory Manual*, 1989). The second series of the filters with imprints were hybridized with a radiolabelled probe corresponding to the mouse *Kin17* cDNA (Angulo et al., N.A.R., 1991, mentioned above).

**b) Hybridization of the recombinant DNAs and detection of the phages which hybridize with the probe.**

Six filters were each placed in a tube and incubated for 3 hours at 65°C in the hybridization solution (5 X SSPE, 5 X Denhardt's solution, 0.5% SDS), in the presence of 50 µg/ml final of sonicated herring sperm DNA (previously denatured at 100°C, for 10 min., then placed in ice).

**b-1)- Radioactive labelling of probes:** 30 ng of DNA corresponding to the 1000-bp fragment of the <sup>HS</sup>*Kin17* cDNA (probe-1000 of sequence SEQ ID NO. 4) or corresponding to the mouse *Kin17* cDNA (probe-1400) were heated for 10 min. at 100°C and incubated in the presence of primers (hexamers), of a deoxyribonucleotide labelled with radioactive phosphorus ([α-<sup>32</sup>P]-dCTP), of three other nonradioactive deoxyribonucleotide triphosphates and of the Klenow fraction of the *E. coli* polymerase I. The labelling is carried out at 37°C for 30 min. and then the probe is purified through a molecular sieve (Sephadex G50, Pharmacia). The level of incorporation of the radioactivity is measured using a liquid scintillation counter. The specific activity of the probe-1000 and probe-1400 was 0.8-3×10<sup>8</sup> cpm/µg (*Random Primed DNA*

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Labelling Kit. Boehringer).

**b-2)- Hybridization:** The filters are incubated in the hybridization solution into which has been added the radiolabelled probe-1000 (SEQ ID NO. 4) or probe-1400 (previously denatured at 100°C for 10 min.). The incubation is carried out for 16 hours at 65°C. After hybridization, the filters are washed in the following way:

3 washes of 10 min. in 2 X SSC buffer, 0.1% SDS at room temperature,

1 wash of 45 min. in 1 X SSC buffer, 0.1% SDS at 65°C.

After removal of excess radioactivity, the filters are placed in contact with X-OMAT AR films (Kodak), which are placed at -80°C for 16 hours.

**c) Isolation of the phages which possess the *HSKin17* gene cDNA.**

The lysis plaques which gave a positive result by autoradiography were located on the Petri dishes, sampled and resuspended in 100 to 300 µl of SM buffer (0.1 M NaCl,  $10^{-3}$  M MgSO<sub>4</sub>, 0.02 M Tris-HCl pH 7.5, 0.01% of gelatin) containing 5 µl of chloroform. The same protocol as that used for the screening makes it possible to subsequently purify the positive phages and their DNA, to determine the size of the inserts and the sequence of the cDNA.

**Purification of the λgt11 phage DNA and determination of the nucleotide sequence of the *HSKin17* gene complementary DNA.**

Procedures are carried out as described in French patent No. 2,706,487.

**Example 3: Methods for characterizing the expression of the *Kin17* gene by detection of the messenger RNA and of the protein encoded.**

**Immunodetection of the *kin17* protein (mouse and human) in cells in culture.**

The cells cultured on coverslips are washed 3 times with PBS, and then fixed using a methanol/acetone solution (3v/7v) for 10 minutes at -20°C. The immunodetection is then carried out at room

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temperature in a humid chamber. After rehydration of the cells for 10 min. in PBS, the coverslips are incubated for 10 min. in a solution of 3% of H<sub>2</sub>O<sub>2</sub> in PBS. After 3 5-min. washes with PBS, the coverslips are incubated for 30 min. in 5% of goat serum in PBS. The coverslips are then rinsed 3 times 5 min. in PBS, and then incubated for 2 hours with an antibody directed against the kin17 protein, termed pAb2064 (Biard et al., Arch. Dermatol., 1997, mentioned above) or an anti-recA antibody, diluted at 1/100 in PBS. After 3 5-min. rinses in PBS, the coverslips are incubated with a biotinylated anti-rabbit immunoglobulin antibody, produced in goats, diluted at 1/200 in PBS. After 3 5-min. washes in PBS, the coverslips are covered for 30 min. with the ABC reagent (Vectastain, Elite ABC Kit, Vector Laboratories), which contains avidin and biotinylated horse radish peroxidase, then rinsed again 3 times 5 min. in PBS. The peroxidase is revealed with diaminobenzidine (Polysciences Inc.), used at the concentration of 0.5 mg/ml in the presence of 0.01% of H<sub>2</sub>O<sub>2</sub> in Tris buffer pH 7.4. The reaction is stopped after a few minutes by immersing the coverslips in water. The coverslips are then mounted with improved Aquamount (BDH, Gurr) and observed using a Carl Zeiss Axiophote 2 microscope equipped for indirect immunofluorescence and with a cooled camera (CCD camera, Coolview, Photonic Science, UK) which is controlled by a computer.

***Detection of the messenger RNA of the <sup>hs</sup>Kin17 by in situ hybridization in T lymphocytes.***

The human lymphocytes, placed onto the slides by cytopsin, were fixed with 4% paraformaldehyde for 10 min., rinsed with PBS, and then dehydrated by successive passages in solutions of alcohol at 70%, 90% and 100%, before being stored at -80°C. After hydration in PBS, all the detection steps were carried out in a humid chamber. The slides are incubated for 10 min. with a solution of 3% H<sub>2</sub>O<sub>2</sub> in PBS, and then rinsed 3 times 5 min in PBS. The slides are successfully treated

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for 10 min. with solutions of 0.1 M glycine in PBS and 0.3% Triton X-100 also in PBS, before being incubated for 2 hours at 37°C in the prehybridization buffer (50% of deionized formamide, 4x SSC, 1x Denhardt's solution, 0.1 mg/ml of salmon sperm DNA, 0.125 mg/ml of transfer RNA and 0.8% of sarcosyl).

**Labelling of the oligonucleotide with digoxigenin.**

100 pm are used of a 40-nucleotide synthetic oligonucleotide (SEQ ID NO. 16 or 17) which is labelled with Dig-11-dUTP at the 3' end using the Boehringer Mannheim kit (Dig Oligonucleotides Tailing Kit. Ref. 1417231). The probe labelling is controlled with the aid of an alkaline phosphatase-coupled anti-digoxigenin antibody (revelation with a solution of NBT-BCIP).

**Hybridization conditions and revelation of hybrids.**

100 µl of hybridization solution, composed of 4 pmol of labelled probe for 96 µl of prehybridization buffer, were placed onto each slide comprising fixed lymphocytes. The hybridization is carried out at 37°C for 16 hours.

The revelation is carried out using the TSA™ Direct kit (NEN kit Ref. NEL 731). After hybridization, the slides are successively washed 3 times for 10 min. in 2 X SSC, 1 X SSC and 0.5 X SSC buffer at room temperature, and then 3 times for 5 min. in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20 buffer (TNT). The cells are then incubated with a blocking buffer composed of 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl and 0.5% of blocking reagent for 30 min. After blocking, the immunodetection is carried out for 90 min. with a peroxidase-coupled anti-digoxigenin antibody (Boehringer Mannheim, Ref. 1207733). The antibody is used at a dilution of 1/100 in the blocking buffer of the hybridization kit (TSA™ Direct). The incubation is followed by 3 5-min. rinses in the TNT buffer, and then the peroxidase is detected by reacting the fluorescein-coupled tyramide for 5 min. as described by the

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supplier (TSA™ Direct, NEN). After 3 5-min. washes in the TNT, the cells are stained with a solution of  $10^{-3}$   $\mu\text{g/ml}$  of 4',6-diamidino-2-phenylindole (DAPI) for 10 min. The slides are then rinsed in the TNT buffer, before being mounted using Vectashield®, which is a fluorescence-protecting mounting product (Vector Laboratories, Ref. H-1000). The fluorescein is observed at 525 nm and the DAPI at 425 nm using a Carl Zeiss Axiophote 2 microscope equipped for indirect immunofluorescence and with a cooled camera, as specified above.

***Detection of the messenger RNA of the  $m\text{mKin17}$  gene by in situ hybridization in mouse testicles.***

The testicles are removed and immediately embedded at  $-20^{\circ}\text{C}$  in the OCT embedding medium (OCT compound, Tissue-Tek, Miles. Ref. 4583).  $10\ \mu\text{m}$ -thick sections are prepared on a cyrostat at  $-20^{\circ}\text{C}$ . They were used immediately, or frozen and kept at  $-80^{\circ}\text{C}$  until use. The first step is fixation with 4% paraformaldehyde, and then the protocol is identical to that described above for detecting the human gene in T lymphocytes. The probes used for the mouse are the synthetic oligonucleotides: antisense = 5'-CCA GGC CTC TTC TCA CCT GCT CCT CAA TGA ACT TGG CAG T-3' (SEQ ID NO. 17) and sense probe: 5'-ACT GCC AAG TTC ATT GAG GAG CAG GTG AGA AGA GGC CTG G-3' (SEQ ID NO. 16). A specific hybridization is observed in the zygotene spermatocytes (Figure 4).

***Detection of the messenger RNA of the  $h\text{sKin17}$  gene by hybridization of membrane-immobilized RNAs with a radiolabelled DNA probe.***

The amount of Kin17 transcript present in various tissues is determined by the Northern method (electrophoresis, transfer and hybridization of the RNAs). Nylon membranes onto which  $2\ \mu\text{g}$  of polyA<sup>+</sup> RNA of various human tissues have been transferred (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testicle, ovary, small intestine, colon and peripheral blood lymphocyte

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(MTN, Clontech)) are used. The prehybridization and the hybridization are carried out in an oven (hybridization oven/shaker from Amersham). The membrane is placed in tubes and incubated at 42°C for 5 hours with 15 ml of hybridization solution (50% formamide, 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS) in the presence of 50 µg/ml final of sonicated herring sperm DNA (previously denatured at 100°C for 10 min., and then placed in ice). The membrane is then incubated in the hybridization solution into which has been added the radiolabelled probe-1000 (SEQ ID NO. 4) (previously denatured at 100°C for 10 min.). The incubation is carried out for 16 hours at 42°C. To remove excess probe, the membrane is washed twice 20 min. at room temperature in 2 X SSC, 0.1% SDS, twice 15 min. at 42°C in 0.5 X SSC, 0.1% SDS, and then once 15 min. at 60°C in 0.1 X SSC, 0.1% SDS. The filters are then placed in contact with X-OMAT AR films (Kodak) or Hyperfilm-MP (Amersham) at -80°C for 60 to 100 hours. Panel A of Figure 3 shows the autoradiograph obtained after hybridization of the total RNAs extracted from various human tissues with the probe-1000. A preferential expression of the *HSKin17* gene exists in certain tissues such as the testicle, the ovary, the heart, the skeletal muscle or the small intestine, whereas it is practically undetectable in other tissues such as the kidney, the lung or the brain.

**EXAMPLE 4: Detection of the *HSKin17* RNA in human tumour lines.**

The level of *HSKin17* messenger RNA in various cells derived from human tumours (promyelocytic leukaemia (HL60)), cervical gland adenocarcinoma (HeLa S3), chronic myeloid leukaemia (K-562), lymphoblastic leukaemia (MOLT-4), Burkitt Raji's lymphoma, colorectal adenocarcinoma (SW480), lung carcinoma (A549 and melanoma (G361)) was determined. The protocol for detecting the *HSKin17* transcript used is the same as that described for detecting the *Kin17* RNA in the human tissues (see Example 3). A membrane onto which 2 µg of

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polyA<sup>+</sup> RNA of various tumour cells have been immobilized (MTN, Clontech) is used. Panel B of Figure 3 represents the autoradiograph obtained after hybridization of the probe-1000 with the total RNAs extracted from various human tumour cells. After quantification of the signals obtained for the *HSKin17* RNA with respect to those calculated for actin RNA, it is observed that the expression of the *HSKin17* gene is very variable according to the line: a very weak expression is observed in the HL60 cells, whereas a difference of a factor of 15 in the K-562 cells or of a factor of 10 in the cells derived from the colorectal adenocarcinoma is observed. The tumour cells thus appear to regulate the expression of the *HSKin17* gene differently.

**EXAMPLE 5:** Study of the transient overexpression of the mouse kin17 protein (*Mmkin17*) and of its truncated forms in human cells.

It has been observed that BALB/c 3T3 fibroblasts, when they are stimulated to proliferate, show an intranuclear accumulation of the *Mmkin17* protein in the cells in S phase (DNA replication). To better define the role of the kin17 protein in cell proliferation, the effect of an overexpression of the *Mmkin17* protein on cell proliferation was tested. A transient transfection system, which makes it possible to overexpress the *Mmkin17* protein in mammalian cells in culture, was used. The vectors obtained correctly express the *Mmkin17* protein (verification by indirect immunofluorescence techniques with the aid of the polyclonal antibodies pAb2064 directed against the *Mmkin17* protein (Biard et al., Arch. Dermatol. 1997, mentioned above and French patent No. 2,706,487)).

The effect of the transient overexpression of various truncated forms of the kin17 protein on cell proliferation is observed.

**Construction of eukaryotic expression vectors.**

All the plasmids used for this study were constructed starting from the vector pCMVDT21, which allows a high expression of the transgene (Bourdon et

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al., 1997). The open reading frame of the mouse *Kin17* cDNA (Angulo et al., 1991, N.A.R., mentioned above) was inserted into the vector pCMVDT21 digested with the restriction enzyme *XhoI*, to obtain the plasmid pCMVKin17. The cDNA termed *mmKin17ΔCT* is also used. It corresponds to a deletion of the fragment between nucleotide 854 and nucleotide 1034 of the *mmKin17* cDNA, and has already been described (Mazin et al., 1994, mentioned above). This cDNA encodes a protein which is truncated in its C-terminal region, termed *kin17ΔCT* protein (deleted in the C-terminal region), and which has a molecular weight of 32407 Daltons. This *Kin17ΔCT* cDNA is inserted into the vector pCMVDT21; the plasmid pCMVKin17ΔCT is thus obtained. A second mutant is obtained by deleting the *mmKin17* cDNA from nucleotide 412 to nucleotide 705. Such a nucleic acid encodes a protein which is truncated (absence of 99 amino acids between residues 129 and 228) at a region containing the sequence which is homologous to the *recA* protein. This mutated protein has been termed *mmkin17ΔHR* (deleted in the Homologous Region). The cDNA *mmKin17ΔHR* cDNA is generated in the following way:

a) PCR amplification of the 5' region of the *mmKin17* cDNA (between nucleotides 1 to 411), from the vector pCD2Kin17 (Angulo et al., 1991) using the pair of oligonucleotides:

5'-AAGCTGCTGCAGCAGCTTATCGGG-3' (SEQ ID NO. 29) and  
5'-GGTACCTTTACACAAGCCCTCTCGCC-3' (SEQ ID NO. 30).

b) PCR amplification of the 3' region of the *mmKin17* cDNA (between nucleotides 706 to 1352) using the primers:

5'-GGTACCACTGCACTGAAGCTGCTGGGG-3' (SEQ ID NO. 31) and  
5'-ATTTACCCAATACTTCACTA-3' (SEQ ID NO. 32).

The two amplification products are then mutually ligated at the *KpnI* site (underlined sequence). The sequencing of the junction of the two fragments showed that the reading frame of the *kin17* protein is intact. The DNA thus obtained is inserted into the vector pCMVDT21 to give the plasmid

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pCMVKin17ΔHR.

Figure 5 shows the schematic representation of the various proteins expressed. The amino acid sequence is represented linearly. The name of the proteins is indicated to the left of each protein, and the size is mentioned to the right. The region which is homologous to the recA protein (HR, aa 163 to 201) and the Nuclear Localization Signal (NLS, aa 235 to 285) are shown in hatched rectangles. The deleted residues are numbered as a function of their respective position in the sequence of the kin17 protein, and are indicated as discontinuities.

**Transient transfection of expression vectors into human cells.**

The various constructs were transfected into HeLa cells, which are human cells derived from a cervical gland adenocarcinoma (reference ATCC CCL-2). 10% of cells transfected is obtained. The transfection is carried out in the following way: the HeLa cells are seeded in 350 mm-diameter dishes into which a glass coverslip had previously been placed. The cells are incubated in a DMEM medium containing 4.5 g/l of glucose, 10% of foetal calf serum and 1% of penicillin/streptomycin, at a density of  $2 \times 10^5$  cells per dish. 24 hours later, the cells are incubated with the following mixture: for each transfection, 3  $\mu$ g of DNA are mixed with 10  $\mu$ l of 2.5 M  $\text{CaCl}_2$  in a final volume of 100  $\mu$ l. The precipitate is homogenized and added dropwise to 100  $\mu$ l of transfection buffer (274 mM NaCl; 1 mM KCl; 1.5 mM  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$ ; 11 mM D(+)glucose; 25 mM HEPES; adjusted to pH 7.15 and sterilized by filtration). The mixture is incubated for 20 minutes at room temperature and then deposited dropwise onto the cells. The incubation lasts 16 hours at 37°C. The cells are then rinsed 3 times with PBS, and then put back into DMEM medium containing 10% of FCS and 1% of penicillin/streptomycin for 24 hours at 37°C. The transfected human cells are fixed with a methanol/acetone solution (3v/7v) at -20°C for 10 min.

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and then dried at room temperature for 10 min., in order to analyse, by indirect immunofluorescence, the localization of the overproduced  $m_{kin17}$  protein.

**Detection of the kin17 protein by indirect immunofluorescence.**

The fixed cells are rehydrated for 10 min. in PBS, and then incubated in a humid chamber for 1h30 at 37°C with the pAb2064 antibody diluted at 1/100 in PBS in the presence of 3% BSA. After 3 5-minute washes in PBS at room temperature and with shaking, the cells are incubated with the second Cy<sup>TM</sup> 2 fluorochrome-coupled goat anti-rabbit immunoglobulin antibody (Jackson Immuno Research Laboratories), diluted to 1/500, for 45 minutes at 37°C and in the dark. The coverslips are then washed 3 times 10 minutes in PBS and incubated in a solution of  $10^{-3}$  µg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The coverslips are then rinsed with water, before being fixed onto a slide with a mounting product (Glycergel, Dako).

Figure 6 corresponds to a photograph of the HeLa cells transfected with the plasmid pCMVKin17 containing the  $m_{Kin17}$  cDNA under the control of the cytomegalovirus promoter. The labelling of the DNA with the DAPI makes it possible to distinguish the cell nuclei, which are stained blue. The green intranuclear staining corresponds to the indirect labelling of the  $m_{Kin17}$  protein overproduced in the transfected cells.

**The  $m_{kin17}$  protein is localized essentially in discrete intranuclear foci.**

Cells which express a low level of  $m_{kin17}$  protein were detected. The localization is clearly nuclear, and a concentration of the  $m_{kin17}$  protein in intranuclear foci of approximately 0.5 µm in diameter is observed (Figure 7A). When the kin17 protein is expressed in large amount, it forms bigger intranuclear foci with dimensions which are similar to, or even large than, the nucleoli (Figure 7B). This result shows that the kin17 protein produced by the vector pCMVKin17 is expressed, that the pAb2064 antibodies recognize its

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native form and that it presents a nuclear localization. These results confirm that, *in vivo*, the nuclear localization signal is indeed functional. The localization of the  $m_{kin17}$  protein in discrete intranuclear foci appears to reflect the functional compartmentalization of the biological processes which take place in the nucleus. In fact, similar profiles have already been observed for other proteins which are involved either in the multiprotein replication complex or in the transcription complex, or for proteins which intervene in the alternative splicing of mRNAs.

**The overexpression of the  $m_{kin17\Delta HR}$  protein leads to the formation of intranuclear aggregates.**

The vector pCMVKin17 $\Delta$ HR is transfected into the human cells under the same conditions as those described above. The detection of the  $m_{kin17\Delta HR}$  protein by the indirect immunofluorescence method is carried out either using the anti-kin17 antibody (pAb2064) or the anti-recA antibody (French patent No. 2,706,487, Angulo et al., Biochimie, 1991, mentioned above). It is observed that the antibody pAbanti-recA is incapable of detecting the  $m_{kin17\Delta HR}$  protein (Figure 8, panel A; HeLa cells transfected with the plasmid pCMVKin17 $\Delta$ HR and processed for immunodetection with the antibody pAbanti-recA). This demonstrates that the region which is homologous to the recA protein is indeed responsible for the cross-reactivity between the kin17 protein and the pAbanti-recA antibodies. Conversely, the kin17 $\Delta$ HR protein is easily detected using the antibody pAb2064 (Figure 8, panel B; HeLa cells transfected with the plasmid pCMVKin17 $\Delta$ HR and process for immunodetection with the antibody pAb2064). A green nuclear staining is observed which corresponds to the indirect labelling of the kin17 $\Delta$ HR protein overproduced in the transfected cells. The cell nuclei are stained with DAPI. The distribution of the  $m_{kin17\Delta HR}$  protein is different from that of the  $m_{kin17}$  protein. Specifically, the  $m_{kin17\Delta HR}$  protein forms large intranuclear aggregates in all the transfected cells, independently of the amount of

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protein overproduced. This might indicate that deleting the amino acids between 129 and 228 increases the binding of the  $M_{m}kin17\Delta HR$  protein to another nuclear component such as DNA or chromatin. Other biochemical approaches have shown that the solubility of the  $M_{m}kin17\Delta HR$  protein is different from that of the  $M_{m}kin17$  protein.

The presence of the  $M_{m}kin17\Delta HR$  protein produces nuclear morphology deformations (NMDs) in the HeLa cells.

HeLa cells are transfected with the plasmid pCMVKin17 $\Delta HR$ , and then the cells expressing the  $M_{m}kin17\Delta HR$  protein are detected by indirect immunofluorescence. The analysis by phase contrast microscopy shows that 100% of the transfected cells show nuclear morphology alterations (Figure 8, phase contrast). When the cells overproduce the  $M_{m}kin17\Delta CT$  protein, it is impossible to detect this type of NMD (Figure 9). In the case of the production of low amounts of  $M_{m}kin17$  protein, the formation of intranuclear foci is observed, and the cells show a normal nuclear morphology without alterations (as described above). Conversely, the overexpression of a considerable amount of  $M_{m}kin17$  protein leads to the formation of very big intranuclear foci, which resemble the distribution of the  $M_{m}kin17\Delta HR$  protein, and in this case, the cells systematically show NMDs (Figure 7). The fact that 100% of the cells which express the  $M_{m}kin17\Delta HR$  protein have nuclear morphology alterations indicates the dominant phenotype of this mutant.

The NMDs are correlated with an inhibition of DNA replication.

It is demonstrated that the expression of the  $M_{m}kin17$  and  $M_{m}kin17\Delta HR$  proteins produces NMDs and affects nuclear biological processes such as replication. A summary of the results is presented in Figure 10. The  $M_{m}Kin17\Delta HR$  cDNA is introduced into HeLa human cells. After expression of the plasmid, the kin17 protein is detected by indirect immunofluorescence with the aid of

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a rhodamine-labelled antibody (red staining). In parallel, in the same cells, the amount of DNA replication is detected by the incorporation of BrdU (green staining); the genomic DNA contained in the nuclei is visualized by DAPI (blue staining) (Figure 10). The overexpression of the  $m_{kin17\Delta HR}$  protein completely inhibits the incorporation of the BrdU (Figure 10).

**Test for clonogenicity of the cells overexpressing the kin17 protein or mutated forms thereof.**

The plasmids pCMVDT21, pCMVKin17, pCMVKin17 $\Delta HR$  or pCMVKin17 $\Delta CT$  were cotransfected with the plasmid pEGFP-N1 (Clontech) into HeLa cells, which are then incubated for 20 days in a medium containing a selection marker (geneticin). The colonies formed are counted. The number of colonies formed by the cells transfected with the plasmid pCMVDT21 is considered as 100%.

The kin17 protein allows the formation of 10% of colonies. The kin17 $\Delta HR$  protein allows the formation of 20% of colonies.

The kin17 $\Delta CT$  protein allows the formation of 70% of colonies.

The presence of the kin17 or kin17 $\Delta HR$  protein considerably affects the growth of the cells, whereas the kin17 $\Delta CT$  protein has no inhibitory action on separate proliferation.

**Effects of the overproduction of the kin17 $\Delta CT$  protein and role of the C-terminal fragment.**

- Construction of the vectors expressing fusion proteins.

The plasmids used for this study were constructed from the vector pEGFP-C3, which expresses the protein GFP (*Green Fluorescent Protein*, Clontech). The cDNA termed  $m_{Kin17\Delta CT}$  was inserted in phase with the cDNA which encodes GFP. The plasmid pEGFP-Kin17 $\Delta CT$  is thus obtained, which expresses the fusion protein GFP-kin17 $\Delta CT$  which has a molecular weight of 60 kDa. A

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second fusion protein was created by inserting, in phase with the cDNA which encodes GFP, the cDNA, termed *Mm*Kin17NLS-CT, which encodes the nuclear localization signal and the C-terminal portion of the kin17 protein, termed kin17NLS-CT protein. The plasmid pEGFP-Kin17NLS-CT is thus obtained, which expresses the fusion protein GFP-kin17NLS-CT which has a molecular weight of 46 kDa.

- Results:

The detection of the kin17 $\Delta$ CT protein by indirect immunofluorescence shows that this truncated protein has rather a homogenous nuclear distribution with a large decrease in the number of intranuclear foci. Conversely, the overproduction of this kin17 $\Delta$ CT protein never generates intranuclear deformation. In addition, its presence in the cells affects neither the DNA replication nor the cell proliferation. These various results show that the peptide region responsible for the formation of intranuclear foci, for the nuclear morphology alterations and for the inhibition of cell proliferation appears to be the C-terminal region.

To verify this hypothesis, the subcellular localization of the GFP-kin17 $\Delta$ CT (deleted in the C-terminal region) and GFP-kin17NLS-CT (possesses the LNS and the C-terminal region of the kin17 protein) fusion proteins was determined.

Figure 18 shows the detection, *in vivo*, of these two proteins in the HeLa cells; whereas the GFP-kin17 $\Delta$ CT fusion protein presents a diffuse nuclear localization, the GFP-kin17NLS-CT protein essentially shows a localization in the form of nuclear foci which are similar to those formed by the kin17 protein. Thus, these observations demonstrate that the C-terminal region of the kin17 protein is capable of directing a heterologous protein (GFP) into the intranuclear foci, and strongly suggest that it is this region which is responsible for the cytotoxic effects observed during the overproduction of the kin17 protein.

EXAMPLE 6: Production of human cells which have a

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stable expression of the  $m_{m}kin17$  protein. Effect on cell proliferation.

To confirm the effects produced by the transient expression of the  $m_{m}kin17$  protein, cells which continuously express the  $m_{m}kin17$  protein were isolated and the effect of this ectopic expression on the cell survival, the proliferation and the morphology of the cells was determined. The mouse  $m_{m}Kin17$  complementary DNA, carried by "EBV" shuttle vectors, was used to transfect human cells, termed HEK293, in culture. HEK293 cells ("Transformed human embryo kidney cells" HEK-Ad5), established from embryonic kidney are transformed with Adenovirus 5 fragments (Graham F.L. et al., *J. Gen. Virol.*, 1977, 36, 59-72). Cells capable of expressing the  $m_{m}kin17$  protein are selected. It has been presumed that the overproduction of the mouse kin17 protein in the human cells has a biological effect which is very close to the human protein, given the conservation of the sequences observed between human and mouse (Figures 2A and 2B).

**The EBV expression vectors carrying the  $m_{m}Kin17$  cDNA.**

The  $m_{m}Kin17$  cDNA is introduced into expression vectors derived from the Epstein Barr virus (EBV vectors) under the control of a very powerful viral promoter ("human cytomegalovirus immediate-early promoter" or "IE HCMV") or of a heavy metal-inducible promoter (mouse promoter mMT-I of the metallothionine I gene). These plasmids are derived from those already published (Biard et al., *Biochem. Biophys. Acta*, 1992, 1130, 68-74; Biard et al., *Exp. Cell Res.*, 1992, 200, 263-271). These vectors have the following advantages: 1) are maintained as stable episomes (extrachromosomal) in human cells; 2) persist at low copy number per cell (1 to 20 in the established lines); 3) replicate once per cell cycle; 4) segregate between daughter cells as chromosomes; 5) present an extremely low background of spontaneous mutagenesis; 6) do not disturb the functional integrity of the transfected cells; 7) allow

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the selection of the cells which carry them as a result of conferring resistance to hygromycin or to geneticin (G418).

The steps for cloning in the EBV vectors and for analysis of these vectors have already been described (Biard et al., *Biochim. Biophys. Acta*, 1992; Biard et al., *Exp. Cell Res.*, 1992, mentioned above). Among the plasmids constructed, 4 of them have more particularly been studied: the vector pEBVMP<sub>Mm</sub>Kin17 (or pB223) (Figure 11), the vector pEBVMTA (or pB220) (Figure 11), the vector pEBVCMV<sub>Mm</sub>Kin17 (or pB291) and the vector pEBVCMVAS<sub>Mm</sub>Kin17 (or pB291AS into which the <sub>Mm</sub>Kin17 cDNA has been inserted in the "antisens" position). After cloning, the vectors are amplified in the DH5 bacterium and purified on "Qiagen" columns according to the supplier's recommendation. This DNA is then used to transfect the human cells.

**The transfection of human cells.**

The eukaryotic cells were cultured in 6-well dishes containing 2 ml of medium per well. Subsequently, 1 to 2 µg of DNA per well (depending on the cell type) are transfected by calcium chloride precipitation (Biard et al., *Biochim. Biophys. Acta*, 1992; Biard et al., *Exp. Cell Res.*, 1992, mentioned above). The cells are incubated at 37°C overnight, and the medium is replaced with fresh medium without antibiotics. 48 hours after the transfection, the cellular proteins are analysed by the "Western blot" or immunohistochemistry technique as described above (Biard et al., *Rad. Res.*, 1997; Biard et al., *Arch. Dermatol. Res.*, 1997). The purification of the plasmids by the "Hirt" technique allows their characterization by DNA-DNA hybridization or by transformation of DH5α bacteria and amplification (Biard et al., *Exp. Cell Res.*, 1992, mentioned above). The cell cultures are maintained in the presence of 250 µg/ml of hygromycin in the medium for 4 days, and then with 125 µg/ml of hygromycin, or of geneticin (750 µg/ml for 4 days, and then 250 µg/ml), in order to determine clonogenic

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growth and to establish continuous lines.

***Effect of the overexpression of the  $m_{m}kin17$  protein in H1299 tumour cells.***

H1299 cells (ATCC CRL-5803) are human lung epithelial cells which have been established after removal from a patient with a lung carcinoma NSCLC ("non small cell lung cancer"). These tumour cells show an inactivation of the p53 gene. The expression of the  $m_{m}Kin17$  cDNA under the control of the powerful IE HCMV promoter leads to a very considerable decrease in the number of colonies formed 14 days after the transfection, even in the presence of the selection marker (hygromycin or geneticin). Under the same conditions, the expression of the antisense  $m_{m}Kin17$  cDNA allows the establishment of very many clones. As a result, it emerges that an ectopic expression of the  $m_{m}kin17$  protein in H1299 tumour cells leads to a considerable selective disadvantage. It is thus very difficult, or even impossible, to establish lines derived from H1299 cells continuously expressing the  $m_{m}kin17$  protein.

***Production of immortal HEK 293 cells which overexpress the  $m_{m}kin17$  protein.***

These HEK 293 cells present the following properties: 1) out of 4 to 5 fragments of viral genome, integrated into their genome (12% of the left-hand end and one copy of 9% of the right-hand end) (Aiello L. et al., *Virology*, 1979, 94, 460-469), only the transcripts from the left-hand end are detected; 2) transformed nature and loss of contact inhibition; 3) secrete their own growth factors and thus grow in low serum medium; 4) moderate tumorigenicity (15% of nude mice present tumours); 5) transfection efficiency higher than 30% (observed 48 h after the transfection of a vector pEBVCMVlacZ or of a vector pEBVCMVEGFP).

It is observed that 48 h after the transfection of the vector pEBVCMV $m_{m}Kin17$ , a large number of cells express the  $m_{m}kin17$  protein at a very high level. After selection with hygromycin (250  $\mu$ g/ml), a very

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considerable number of clones is observed in all cases, this number always being greater than that observed after transfection of the control vector pEBVCMVAS<sub>Mm</sub>Kin17. Thus, 14 days after the selection, the viability of the cells is compatible with the expression of the <sub>Mm</sub>kin17 protein. After several weeks, a progressive loss of the number of cells expressing the <sub>Mm</sub>kin17 protein is observed. This occurs in many transfection experiments. This indicates that the ectopic expression of the <sub>Mm</sub>kin17 protein leads to a selective disadvantage for the HEK 293 cells, which leads to their disappearance. Conversely, the HEK 293 cells transfected with other EBV vectors, such as the vectors pEBVCMVlacZ, pEBVMTlacZ, or pEBVCMVlacI, maintain a stable expression of the gene of interest (herein, the bacterial genes *lacZ* or *lacI*) for many months, or even many years (Biard et al., *Cancer Res.*, 1992, mentioned above).

HEK 293 cells are transfected with the vector pEBVMT<sub>Mm</sub>Kin17. In this vector, the expression of the <sub>Mm</sub>Kin17 cDNA is controlled by the mMT-I promoter. As a result, the basal expression of the <sub>Mm</sub>Kin17 cDNA is considerably lower than that obtained with the IE HCMV promoter. In addition, the expression can be increased using heavy metals in the culture medium. Several stable clones which express a very low level of <sub>Mm</sub>kin17 protein have been isolated and analysed. The advantage of this system is that the introduction of 100 µM of Zn and 1 µM of Cd in the culture medium activates the mMT-I promoter and increases the expression of the <sub>Mm</sub>Kin17 cDNA (Figure 12).

One clone, termed B223.1 cells (Figure 12) since it carries the vector pEBVMT<sub>Mm</sub>Kin17 (or pB223), presents quite a high basal expression of the <sub>Mm</sub>kin17 protein, whereas the clone termed pB223.2 presents a low basal expression of the <sub>Mm</sub>kin17 protein. The characterization of this clone for more than 8 months in continuous culture has shown that the level of the <sub>Mm</sub>kin17 protein is correlated with a very considerable

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decrease in cell proliferation.

The  $m_{kin17}$  protein is detected in the transfected cells by immunocytochemical staining; whereas no signal is observed with the B220 cells (Figure 12C and D), an intense signal is detected in all the B223.1 cells, 24 hours after a treatment with heavy metals, using an anti-recA antibody (Figure 12A and 12B).

Differences in expression are also observed in the B223.1 cell population.

Using conventional microscopy with a narrow filter to analyse the emission of the Cy2<sup>TM</sup> fluorochrome, less than 1% of B223.1 cells are observed overexpressing the  $m_{kin17}$  protein in the absence of stimulation with heavy metals (Figure 13A); after stimulation with heavy metals, a strong specific signal is detected, located in the nuclei of the B223.1 cells (Figure 13B).

Under these experimental conditions, the endogenous  $h_{kin17}$  protein is not detectable (Figure 12C and 13C).

A decrease in the clonogenic growth of the B223.1 cells, an incapacity to grow at low density and a poor adhesion to the culture support are also observed (Figure 13B). The B223.1 cells are often giant and polynucleated (Figure 14). More polynucleated cells are observed in the B223.1 cell population than in the other two cell lines (B220 and B223.2) (Figure 15). They present multilobed nuclear structures and micronuclei. These results indicate that the viability of the human cells HEK 293 is compatible with a low constitutive expression of the  $m_{kin17}$  protein. Conversely, a high expression level negatively affects cell proliferation. These results indicate that the overexpression of the  $m_{kin17}$  protein compromises cell viability. All these results reinforce the hypothesis according to which the  $m_{kin17}$  protein should intervene in the control (negative) of cell proliferation (Figure 16).

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When the B223.1 cells are seeded at a density of  $10^3$  cells/cm<sup>2</sup>, it is observed that, in the absence of heavy metals, these cells are incapable of growing after 7 days in culture; they remain round and do not spread out (Figures 12 and 17A). Whereas the B223.1 cells begin to grow after 7 days in culture, the other two cell lines almost reach confluence (Figure 17A). When these various cell lines are grown at various densities to take into account their efficiency in forming plaques, a reduced rate of proliferation is observed for the B223.1 cells, in comparison with the other two lines (Figure 17B). Since no difference is observed in the presence or in the absence of hygromycin B, it should be considered that these results are not due to a difference in sensitivity to the selection medium (Figure 17B).

As emerges from the above, the invention is in no way limited to those of its modes of implementation, of execution and of application which have just been described more explicitly; on the contrary, it embraces all the variants thereof which may occur to the person skilled in the art, without straying from the context or the scope of the present invention.

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